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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research training program is to produce highly qualified scientists for careers as independent investigators in the field of breast cancer. During the last 25 years, there has been a fundamental revolution in the understanding of molecular and cell biological concepts related to cell growth, function and tumorigenesis. To utilize what has been learned and to continue future progress in the area of breast cancer requires the continued availability of well-trained, innovative and committed scientists. This program represents an interdepartmental training program involving 15 investigators from seven departments. Trainees are predoctoral and postdoctoral fellows with backgrounds in biochemistry, cell and molecular biology, molecular genetics and molecular virology. The training program provided trainees with additional foundation in carcinogenesis and breast cancer. In addition to the core curriculum taken by the predoctoral fellow in their respective academic departments, program enhancement is provided through trainees' participation in a graduate course on "Molecular Carcinogenesis" (predoctoral fellows), a Breast Disease Research Seminar (all trainees) and participation at national meetings and local seminars. Predoctoral and postdoctoral trainees are enrolled in the program.

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Table of Contents

INTRODUCTION

Breast cancer is a complex disease whose ultimate understanding will require the integration of facts resulting from a multidisciplinary approach. Continued basic science research will provide a fuller understanding of the basic mechanisms of breast cancer that is necessary to conquer the disease in humans. In order to have the scientific human armamentarium to further this understanding, this training grant focuses on producing qualified scientists for careers as independent investigators in the area of breast cancer. The rationale for a targeted training grant in breast cancer is based on the belief that the elucidation of how oncogenes, tumor suppressor genes, hormones and growth factors act at the molecular level and as developmental-specific agents are critical questions directly relevant to the etiology, prevention, diagnosis, treatment and prognosis of human breast cancer. The training program has drawn together individuals who have an established research and training background in the mammary gland with individuals who have a research and training background in cell biology, molecular endocrinology, molecular biology, molecular virology, viral oncology, molecular genetics and biochemistry. The strength of the program is two-fold. First, the program gathers together members of diverse disciplines to focus on the training of predoctoral and postdoctoral students for careers in an area that, by its biological nature, is multi-disciplinary. Second, the program introduces new intellectual approaches and insights to the problem of breast cancer that will be continued by the next generation of research scientists.

The design of the training program provides for trainees to be exposed to clinical problems and recent advances as well as the multi-disciplinary approaches to answering fundamental questions related to breast cancer research. The familiarity and close proximity of the training faculty facilitates and encourages the development of a new generation of research scientists who will be able to understand the problem of breast cancer at a more complex level and from a multi-disciplinary orientation.

BODY

a. Trainees

The goal of this training program is to provide an environment for training in breast cancer research. To foster this goal, candidate graduate students have to meet a minimum set of requirements. Graduate students have to be at least in their second year of graduate school and have selected a thesis problem focusing on an aspect of mammary gland growth, differentiation and/or cancer. These students are supported for two years by the training program provided they maintain satisfactory progress in their research program and they participate in the weekly "Breast" seminar and attend the course "Molecular Carcinogenesis". The postdoctoral fellows are supported by the training program for two to three years provided they maintain satisfactory progress in their research project and actively participate in the weekly "Breast" seminar. The five fellows from the grant year 2000 - 2001, their departmental affiliation, mentor, research problem and an Abstract of their research is provided below.

b. Research Projects

1) Geetika Chakravarty, Ph.D., Department of Molecular and Cellular Biology; Mentor, Jeffrey Rosen, Ph.D., Professor; "p190-B in mammary development and cancer."

The overall objective of this fellowship was to establish that p190-B expression is critical for ductal morphogenesis and that its aberrant expression facilitates cancer progression. Functional studies of p190-B that are being pursued to address these objectives are described in the following section.

RhoGAP-p190-B is essential for mammary development: As reported previously, loss of both alleles of p190-B resulted in a complete failure of outgrowth of the transplanted mammary anlage, and approximately a 50% decrease in the take rate for the heterozygous embryos. In addition, a statistically significant decrease in the rate of ductal outgrowth(s) as compared to wildtype mice was observed for p190-B heterozygotes at four and five weeks of age. This defect was rescued by six weeks of age. One possible explanation for this rescue was that at 4 to 5 wks after birth when these mice have attained sexual maturity, a burst of epithelial proliferation rescues the phenotype so that by six weeks of age the extent of outgrowth is similar in the heterozygotes and wildtype mice. To test this hypothesis, levels of proliferation in the outer two cell layers of the TEBs in *p190-B* hetrozygotes and wild type mice were compared (Figure 6A). Highly statistically significant differences now were detected ($p \leq 0.000007$) in proliferation in the two outer most cell layers of the TEBs of heterozygous **(4.7% ± 1.5)** as compared to those of the wild type **(37.3%± 2.5)** mice at 5 weeks of age.

p190-B-null mammary buds fail to grow out as a result of perturbed epithelial-mesenchymal interactions. These studies indicated that the size of the mammary bud was decreased in the p190-B null embryos and that the mesenchyme surrounding the bud was disorganized. Retarded embryonic mammary development as a result of improper epithelial-mesenchymal interactions was detected in the p190-B null embryos and to a lesser extent in the heterozygotes as determined using tenascin C as a marker for the mesenchyme, and p63 as a marker of the epithelium, respectively. In addition, preliminary in situ hybridization studies suggest that p190-B may be preferentially expressed in the epithelial bud and that loss of p190-B may result in decreased migration and increased apoptosis of the epithelial cells in the mammary bud, as well as a disruption of inductive signals to the mesenchyme. Additional analysis of the mammary anlage from day E10 to E15 are in progress.

p190-B overexpression results in collapse of the actin cytoskeleton, loss of cell-cell junctions and induction of MMP-2. To study the mechanism by which p190-B overexpression might regulate cell migration and breast cancer progression, we wanted to generate both stable cell lines and transgenic mouse models. Preliminary attempts to generate stable cell lines expressing p190-B indicated that use of a regulatable system would be preferable to be able to directly relate the phenotype observed in stable cell clones to the levels of p190-B expression. A few stable clones expressing p190-B were generated using MCF10AT normal breast cells and MDA-435 breast cancer cell lines. The preliminary examination of these clones indicated that the p190-B expressing clones exhibited a complete loss of the actin stress fibers, as well as the loss of well defined cell-cell junctions. In addition they and less adherent and therefore potentially more invasive. Consistent with these latter findings, the induction of the matrix metalloprotease, MMP-2 was detected in cells overexpressing p190-B.

To investigate the role of p190-B in breast tumor progression, recent studies are aimed at generating a tet-regulatable transgenic and a cell culture model.

2) Fabrice Petit, Ph.D., Department of Molecular and Cellular Biology; Mentor, Sophia Tsai, Ph.D., Professor; "Role of COUP-TFII in mammary gland development and tumorigenesis."

To study the physiological function of COUP-TFII (Chicken Ovalbumin Upstream Promoter-Transcription Factor II) *in vivo*, we have generated null mice for this gene. The mutant mice die during the early embryonic development around E10.0 days of gestation from heart and vasculature defects. It has been shown that Angiopoietin-1 (Ang1), a potent angiogenic factor, is down-regulated in COUP-TFII null mice (Pereira *et al.*, *Genes & Dev.*, 13, 1037-1049, 1999). Interestingly, COUP-TFII is highly expressed in mammary gland and in many breast cancer cell lines. So far, no physiological role has been described for COUP-TFII in this organ. Nevertheless, it has been reported that COUP-TFII can either inhibit or enhance the transactivation function of transcription factors such as ER, through a direct interaction. It is possible that COUP-TFII may play a role in the development and/or the function of the mammary gland. Since COUP-TFII null mutants show defects in angiogenesis, which plays key roles in tumor growth and metastasis, it is possible that COUP-TFII plays a role in the tumorigenesis and, particularly, in the breast cancer. For these reasons, my project is to use COUP-TFII null mutants to study its role in the mammary gland development and the breast cancer formation. Since the COUP-TFII null mice die prior to mammary gland formation, it does not allow us to analyze the physiological function of COUP-TFII during organogenesis.

To rescue the COUP-TFII null mutant lethality, we used the Ang1 promoter to drive the expression of COUP-TFII. A fragment of 10.5 kb of the angiopoietin-1 gene has been cloned from a Bacterial Artificial Chromosome (BAC) and used to specifically knock in the endogenous Ang1 gene. For this purpose, The COUP-TFII cDNA is inserted into the first exon, in frame with the Ang1 first codon. To analyze the expression of COUP-TFII, an IRES-lacZ construct is introduced. Homologous recombination was identified by mini-Southern. 45 positive targeted clones were isolated and 9 of them have been injected into blastocysts isolated from a C57BL/6 mouse mother. Three independent mouse lines were established. By using X-Gal staining, we demonstrated that this gene expressed highly in the heart at early embryonic stages as expected. To rescue the COUP-TFII null mutant, double heterozygotes (COUP-TFII$^{+/-}$; Ang1$^{COUP\text{-}TFII/+}$) were generated. We intercrossed these double heterozygotes to generate COUP-TFII$^{-/-}$; Ang1$^{COUP\text{-}TFII/+}$ mice. Unfortunately, among 300 embryos we analyzed, we could not get any embryo older than 10 days with a COUP-TFII$^{-/-}$; Ang1$^{COUP\text{-}TFII/+}$ genotype. Therefore, we were unable to rescue the COUP-TFII null mice by knocking-in COUP-TFII under the control of Ang1 promoter. Since we could not rescue the COUP-TFII null mutant, it was not possible for us to examine the role of COUP-TFII in mammary gland development and tumor progression. This project has been abandoned and Dr. Petit is now engaged in studies of gene regulation in another organ system.

3) Yue Wei, Department of Biochemistry; Mentor, Wade Harper, Ph.D., Professor; "Molecular dissection of the S-phase transcriptional program controlled by the cyclin E/p220^{NPAT} signaling pathway."

The specific aims of this study are:

Examine the transcription regulation of non-histone genes by p220^{NPAT}. Microarray approaches will be used in conjunction with gain-of-function experiments to identify candidate p220^{NPAT} regulated genes, which will serve as a starting point to more fully understand how p220^{NPAT} controls S-phase events.

Genetic analysis of the cycliln E- p220^{NPAT} pathway. Using the conditional p220^{NPAT} knock-out cell, the phenotypic consequences of loss of p220^{NPAT} will be determined, including whether it is essential for cell division and it so where and how it arrests the cell.

Identification of potential functional domains of p220^{NPAT}. Through the functional analysis of a series of p220^{NPAT} mutants and partial proteolysis, the potential functional domains will be determined.

The results of these experiments are:

Establishment of the stable cells with inducible overexpression of p220^{NPAT} and microarray analysis. U2OS cells were established with doxycyclin-inducible overexpression of p220^{NPAT} (U2OSTetON-NPAT). U2OSTetON-NPAT and U2OSTetON cells were collected before and after doxycycline added into the media at multiple time points. Samples from each time point were analyzed with western blot for protein level and with flow cytometry for cell cycle, showing overexpression of p220^{NPAT} and S-phase promotion in U2OStetON-NPAT cells with doxycycline. Luciferase activities from histone H4 promoter were also induced in U2OStetON-NPAT cells with doxycycline. RNAs from samples from 12h to 32h after induction as well as control (without induction) have been sent for microarray analysis and are currently being processed by Millennium Pharmaceutical Inc.

Phenotype of HCT116 p220^{NPAT} conditional knock-out cells. On the base of HCT116 p220$^{NPAT-/E2flox}$ cell, the stable HCT116 p220$^{NPAT-/E2flox}$ER-Cre was established. Through the addition of 4-hydroxytamoxifen (OHT), exon 2 of npat on the flox-alleles was looped out, determined by PCR-genotyping. It was found both two HCT116 p220$^{NPAT-/E2flox}$ER-Cre clones tested lost the potential to form colonies with OHT but not in control cells. By flow cytometry analysis, apoptosis was detected in p220$^{NPAT-/E2flox}$ER-Cre cells.

Identification of potential functional domain of p220^{NPAT} through "lox-P scanning mutagenesis and partial proteolysis. A method was developed to systematically mutate NPAT by replacing amino acids from this protein with identical number of amino acids of loxP. Through this method twelve mutants were generated. These mutants are being characterized in terms of their activity (transcription activity, localization and cell cycle). At the same time, Flag-p220^{NPAT} was purified from insect cells and partial proteolysis is being performed with multiple proteases. All experimental approaches are ongoing.

4) Michelle Martin, Baylor Breast Center, Department of Molecular and Cellular Biology; Mentor, Peter O'Connell, Ph.D., Professor; "The metastasis gene on chromosome 14q."

This project involves the characterization of a region of chromosome 14q in breast cancer. Interest in this region came about after previous loss of heterozygosity (LOH) uncovered a unique genetic event on chromosome 14q. Unlike all other markers tested, loss of marker D14S62, through LOH, was associated with node-negative disease and slower spread to distant sites. Mapping of MTA1 (metastasis associated 1), show that it mapped to YAC859D4, which was inside our region of interest. We produced a polyclonal antibody to use in immunoblot and immunohistochemical (IHC) studies.

Characterization of Candidate Gene MTA1. Western blot studies on human breast tumors were carried out with our MTA1 antibody. Two sets of primary breast tumors were studied; one node-positive (n=315), and the other a mixed node-positive/node-negative set (n=48). In the node-positive set, MTA1 was found to correlate with several other breast cancer-related factors such as ER (p= <.0001), NCOR (p= .0001), AIB1 (p= <.0001), FKHR (p= <.0001), and SAFB (p= <.0001). Cell fractionation studies on MTA1 have been completed on different breast cancer cell lines including MDA-MB-231, MCF7, MDA-MB-435, and T47D. These studies support the notion that the MTA1-associated signal detected by the polyclonal antibody is nuclear in origin. These results are currently being written up for submission to the Journal of the National Cancer Institute.

This observation that the MTA1 signal is nuclear then guided our interpretation of immunohistochemistry (IHC) performed with our MTA1 antibody on tissue arrays containing node-positive and node-negative primary breast tumors from patients with long-term follow-up data. Results confirmed the correlations seen in the western study. MTA1 correlated with ER (p=<.0001) and PgR (p=.0830). Suprisingly, MTA1 expression did not correlate with the presence of positive nodes, a known marker for the presence of metastasis. In order to classify MTA1 experession, a cutpoint was used to classify MTA1 expression as either "low" or "high". Most interestingly, in both univariate and multivariate analyses using this cutpoint, high levels of MTA1 were found to correlate strongly with a decrease in disease-free survival (997 patients, 326 recurrences). Since MTA1 did not seem to correlate with positive nodes, we decided to classify these tumors into all node-negative (577 patients, 128 recurrences) cases. Also, since this analysis was done on a mixed population of untreated and treated patients, we decided to perform analyses on just the node-negative, untreated population (373 patients, 97 recurrences), to remove any bias or influence on MTA1 expression created by treatment. In both of the subsequent analyses, high levels of MTA1 remained statistically significant with a decrease in disease free survival. The only factor in multivariate analyses that was a stronger predictor for decrease in disease-free survival was the presence of positive lymph nodes. However, none of the correlations seen with MTA1 extended to overall survival. As a result, MTA1 IHC could augment the current practice of axillary dissection for disease staging, and could be an especially useful prognostic factor in the axillary node-negative setting. This is important because although many women present with axillary node-negative disease, up to 30% of them will recur with distant metastases within 5 years. We hypothesize that MTA1 does not correlate with positive nodes because MTA1 serves more as a marker of systemic metastatic disease than as a marker of nodal metastasis.

The results of the clinical IHC study are being written up for submission to Nature Medicine. Michelle will be defending her thesis research in September, 2002.

5) Isabel Latorre, Department of Molecular Virology and Microbiology; Mentor, Larry Donehower, Ph.D., Professor; "Examining roles for *MUPP1, MAGI-1, ZO-2* and *Dlg* in Mammary Carcinogenesis"

Human adenovirus type 9 (Ad9) generates estrogen-dependent mammary tumors in rats, and the primary oncogenic determinant of this virus is its E4-ORF1 oncoprotein. A PDZ domain-binding motif present at the carboxyl-terminus of Ad9 E4-ORF1 is required for it to bind to a select group of cellular PDZ proteins, including MUPP1, MAGI-1, ZO-2 and Dlg, as well as to transform cells and to promote mammary tumors in animals. This motif has also recently been shown to be necessary for Ad9 E4-ORF1 to stimulate the phosphoinositide 3-kinase (PI 3-K) pathway, which is deregulated in many different human cancers. Whether Ad9 E4-ORF1 must bind to one or more of the PDZ proteins in order to activate PI 3-K is not yet known. Although the specific functions of the Ad9 E4-ORF1-associated PDZ proteins have not been determined, mammalian Dlg is a functional homologue of the *Drosophila* dlg tumor suppressor protein and Dlg over-expression blocks progression of mammalian cells from G_0/G_1 phase to S phase of the cell cycle. Recent findings likewise suggest that ZO-2 is a tumor suppressor protein, as its expression was reportedly lost or significantly decreased in the majority of examined human breast adenocarcinomas, as well as in other human malignancies. Interestingly, we have evidence suggesting that MAGI-1 can bind to and enhance the activity of the tumor suppressor PTEN, an antagonist of PI 3-K. Thus, the aims of my thesis project are (Aim 1) to determine whether targeted disruption of *MUPP1*, *MAGI-1*, *ZO-2* and *Dlg*, alone or in combination, predisposes mice to proliferative disorders or cancers and (Aim 2) to examine whether cells derived from the knockout mice display increased susceptibility to transformation by various oncoproteins and/or whether Ad9 E4-ORF1 retains the ability to activate the PI 3-K pathway in these mutant cells.

Targeted disruption of PDZ-protein genes in mice. As mice having a mutation within the *Dlg* locus were recently reported by Caruana *et al,* I have obtained these animals for utilization in experiments planned in Aim 2. This *Dlg* mutation results in perinatal lethality and craniofacial abnormalities, underscoring the crucial role of Dlg in mammalian development.

Eight male chimeras, derived from the mutant *MAGI-1* ES cell clone 4H8, were crossed with C57BL/6 females. I screened the resulting offspring by PCR and Southern blot analyses and identified six mice that carry one mutant *MAGI-1* allele. These heterozygous mutant *MAGI-1* mice are being crossed with each other to obtain homozygous mutant *MAGI-1* mice and embryo fibroblasts. I am performing additional crosses to increase heterozygous mutant *MAGI–1* mouse numbers and to introduce the *MAGI-1* mutation into the well-characterized C57BL/6 genetic background.

I constructed a new *MUPP1* targeting vector, which has been electroporated into ES cells by our collaborator Dr. Steve Jones at the University of Massachusetts. Dr. Jones will send me DNA from drug-resistant ES clones, which I will screen by PCR and Southern blot analyses for homologous recombination events.

I obtained a *ZO-2* mutant ES cell clone from Dr. Skarnes at BayGenomics, a consortium of research groups in San Francisco funded as part of the Programs for Genomic

Applications of the National Heart, Lung, and Blood Institute. These mutant cells are predicted to express a truncated ZO-2 protein, containing half of PDZ domain 1 and lacking the other two PDZ domains as well as the SH3 and GUK domains, fused to LacZ. Dr. Jones has injected these cells into blastocysts and obtained chimeric offspring, which he will send to me so that I may determine whether these animals can transmit the mutant *ZO-2* gene through their germline.

Assessing a possible role for the PDZ proteins in PI 3-K signaling. In collaboration with Dr. Monica Justice, I obtained mutant MEF having a large chromosome 4 deletion that removes *MUPP1*, in addition to other genes. My preliminary results indicate that, compared to wild-type MEF, MEF unable to express MUPP1 display enhanced activation of PI 3-K signaling by Ad9 E4-ORF1. These mutant *MUPP1* MEF lines stably expressing Ad9 E4-ORF1 also display an enhanced transformation phenotype compared to control MEF that express MUPP1. I am presently attempting to reintroduce *MUPP1* into the mutant MEF in an attempt to reverse this effect, as well as testing whether other oncogenes similarly exhibit enhanced transforming potential in MEF unable to express MUPP1.

KEY RESEARCH ACCOMPLISHMENTS

The major results of the past year in bullet form are:

- P190-B, a RhoGTPase, is required for normal mammary morphogenesis during fetal and postpubertal stages.
- P190-B overexpression results in dysfunctional actin cytoskeleton, loss of cell junctional complexes and increase of MMP-2.
- The loss of $p220^{NPAT}$ function, a cyclin E signaling pathway component, results in loss of colony formation by tumor cells. This loss is, in part, driven by increased apoptosis.
- MTA1, a metastasis associated gene that has been mapped to LOH on chromosome 14q, has been associated with decreased disease free survival in a large study of primary breast tumors using immunohistochemistry. MTA1 is localized in the nucleus and is an independent prognostic factor in node-negative disease.

REPORTABLE OUTCOMES

Enhancement Programs

Three education programs specific for this training program were functional over the past year. The bi-weekly "Breast" seminar included faculty and trainees. The schedule for the seminar series is shown in Table 1.

The second education enhancement program is the Invited Speakers program. This program allows both the faculty and fellows supported by the program to interact with the invited speaker. The three speakers were Dr. Fred Miller (Karmanos Cancer Center, Detroit, Michigan) on xenograft models for human breast premalignant disease, Dr. Jeff Green (National

Cancer Institute, NIH) on mouse models of human breast cancer, and Dr. Carlos Arteaga (Vanderbilt Cancer Center) on cell cycle dysregulation in experimental and human breast cancer.

The third education enhancement program is the course in "Molecular Carcinogenesis," which is given every Winter bloc and each predoctoral trainee is required to pas. This course is organized by Dr. Larry Donehower and the teaching faculty includes Drs. Medina, Harper, Donehower and Brown.

Trainee Review

With respect to trainee review, there are three turnovers for the new year (2002 – 2003). Michelle Martin has finished her Ph.D. research and will defend her thesis in September, 2002. Yue Wei now has an individual fellowship and is expected to finish her Ph.D. research in late 2003. Fabrice Petit is now supported by another research mechanism as his project could not be concluded due to technical reasons. The three new trainees are Bonnie Nannenga, Dept. of Molecular Virology and Microbiology, Mentor, Larry Donehower, Ph.D., Professor; Harry Toumazou, Breast Center, Mentor, Adrian Lee, Ph.D., Assistant Professor; and Yang David Lee, Department of Biochemistry, Mentor, Stephen Elledge, Ph.D., Professor.

Publications

Three of the supported trainees have publications in leading scientific journals. These are listed below and are provided as appendices.

1. p190-B RhoGAP is essential for mammary morphogenesis. Geetika Chakravarty, William Buitrago, Jeffrey Settleman and Jeffrey M. Rosen (Submitted)

2. Molecular genetic analysis of Rho-GTPase activating protein p190-B function in mammary gland development and in breast cancer. Geetika Chakravarty & Jeffrey Rosen, Department of Cell Biology, Baylor College of Medicine, Houston, Tx 77030-3498. (GRC Cancer conference, New port, Rhode Island, July 2001)

3. A novel RhoGAP p190-B is essential for mammary morphogenesis. Geetika Chakravarty, William Buitrago, Jeffrey Settleman and Jeffrey M. Rosen, Dept. of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030 USA. Massachusettes General Hospital Cancer Center and Harvard Medical School, Charlestown, MA 02129, USA. (GRC Mammary Gland Biology Conference, Il Cicco, Italy, April 2002.

4. Salas, R., Petit, F.G., Pipaon C., Tsai, M.J. and Tsai, S.Y. Induction of chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) gene expression is mediated by ETS factor binding sites. Eur J Biochem. 269:317-325, 2002.

5. Martin, M.D., Fischbach, K., Osborne, C.K., Mohsin, S.K., Allred, D.C., and O'Connell, P. Loss of heterozygosity events impeding breast cancer metastasis contain the *MTA1* gene. Cancer Res 61:3578-3580, 2001.

FLOOD LOSSES

The flood due to Tropical Storm Allison in June 2001 affected the research program of Dr. Geetika Chakravarty. The mice (p190B) lost to the storm have been bred and new weanlings accumulated to allow continuation of the previously planned experiments. Lost reagents, mice and time were costly and had a temporary negative impact on the research.

CONCLUSIONS

The training program in breast cancer is functioning as planned and major alterations are not planned at this time. The inclusion of faculty from the Baylor Breast Center as participating faculty has added a translational dimension.

APPENDICES

Table 1.
Publications.

Table 1
Breast Disease Research Group
2001-2002 Schedule
Wednesdays 12:00 p.m., Room M616 (Debakey Bldg.)

DATE	NAME	TITLE	DEPARTMENT
09/19	Suzanne Fuqua, Ph.D.	Professor	Breast Center
10/03	Mike Lewis, Ph.D.	Assistant Professor	Breast Center
10/17	Debananda Pati, Ph.D.	Assistant Professor	Pedi-Hematology-Oncology
10/24	Powel Brown, M.D.	Associate Professor	Breast Center
10/31	HALLOWEEN PARTY		
11/14	Stacey Bussell	Graduate Student	Molecular & Cellular Biology
11/21	THANKSGIVING WEEK		
12/12	SAN ANTONIO MEETING		
12/26	CHRISTMAS WEEK		
01/09	Steffi Oesterreich, Ph.D.	Assistant Professor	Breast Center
01/16	Larry Donehower, Ph.D.	Professor	Molec. Virology & Microbiol.
01/30	Dr. Elizabeth Anderson	Senior Investigator	Manchester, England
02/06	Fred R. Miller, Ph.D.	Professor	Karmanos Cancer Inst.
02/20	Jenny Chang, M.D.	Assistant Professor	Breast Center
02/27	Jeffrey E. Green, Ph.D.	Senior Investigator	National Cancer Institute
03/13	D. Craig Allred, M.D.	Professor	Breast Center
03/20	Melanie Ginger, Ph.D.	Postdoctorate Fellow	Molecular & Cellular Biology
03/27	EASTER WEEK		
04/03	Carlos Arteaga, M.D.	Professor	Vanderbilt University
04/10	AACR Meeting		
04/24	Michelle Martin	Graduate Student	Breast Center
05/08	Yue Wei	Graduate Student	Biochemistry
05/15	Rachel Schiff	Assistant Professor	Breast Center

Induction of chicken ovalbumin upstream promoter-transcription factor I (*COUP-TFI*) gene expression is mediated by ETS factor binding sites

Ramiro Salas*, Fabrice G. Petit, Carlos Pipaon†, Ming-Jer Tsai and Sophia Y. Tsai

Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI, or NR2F1) is an orphan nuclear receptor that plays a major role in the development of the nervous system. We show here that three ETS response elements in the *COUP-TFI* promoter mediate its transcription. A reporter gene containing these ETS binding sites is activated by Ets-1, while the same reporter with point mutations on all three ETS response elements is not. We also show that Ets-1 binds to these response elements and that other ETS factors also transactivate the *COUP-TFI* promoter. In addition, COUP-TFI is coexpressed with some ETS factors in the mouse embryo. These results indicate that members of the ETS family can activate *COUP-TFI* gene expression.

Keywords: COUP-TFI; ETS; gene expression; transcription; orphan receptors.

Chicken ovalbumin upstream promoter-transcription factors (COUP-TFs) are among the best characterized orphan members of the nuclear receptor superfamily [1]. COUP-TFs have been shown to be negative regulators of the transcription of many genes [1,2], but can also act as activators of gene transcription [1,3]. In mammals, two *COUP-TF* genes have been identified, *COUP-TFI* and *COUP-TFII*. Although they have different physiological functions [4,5], sequence analysis and molecular studies indicate that they share similar properties. The expression patterns of COUP-TFI and COUP-TFII have been extensively studied in a number of species [6]. In the mouse, COUP-TFI is first detected at the embryonic day 7.5 (e7.5), its expression reaches a peak at e12.5, declines before birth [6] and remains low during adulthood. COUP-TFI null mice die perinatally and exhibit neuronal defects in axonal guidance and arborization [4] and thalamocortical connections [7].

The ETS family of transcription factors is composed of a large number of proteins that share a similar DNA-binding domain (DBD), called the ETS domain [8]. These proteins bind as monomers to a core sequence GGAA/T and activate transcription of promoters having this ETS response element [9,10]. Besides the GGAA/T core sequence, at least three bases, 5′ and 3′, of this core are important for high affinity and specific DNA binding [9]. Although ETS proteins contain activation domains [11], Ets-1, Ets-2 and other ETS proteins need to interact with other transcription factors to transactivate their target genes [12]. These factors include Fos [13], SRF [14], NF-EM5 [15], AP1 [12] and NFAT [16]. The necessity for the accessory factors is likely due to poor DNA-binding affinity of full-length Ets-1 rather than to poor potential of its activation domain. This is supported by the existence (at least in Ets-1 and Ets-2) of an auto-inhibitory domain which, in the absence of accessory factors, prevents Ets-1 binding to DNA [10,17]. When this domain is removed, Ets-1 binds DNA with higher affinity even in the absence of accessory factors. The auto-inhibitory domain is located within exon VII and an alternatively spliced form of Ets-1 that lacks this exon is constitutively active [18].

Little is known about the upstream signals that regulate *COUP-TFI* gene expression. Here we show that Ets-1 and other ETS factors are able to transactivate COUP-TFI expression through a cluster of ETS response elements in the promoter. In addition, several ETS factors colocalize with COUP-TFI in different tissues of the developing mouse embryo.

MATERIALS AND METHODS

Genomic screening

To isolate the *mCOUP-TFI* promoter, a genomic library (129SVJ Mouse Genomic Library in the Lambda FIX II vector, Stratagene) was screened using part of the 5′ UTR of the *mCOUP-TFI* gene. The fragment was labeled with ^{32}P-dCTP by random priming (Prime-a-gene kit, Promega).

The genomic library was used to infect XL-1 blue bacteria and standard protocols were used to perform the screening [19]. After tertiary screening, the phage DNA was isolated using the λ Wizard kit (Promega), cut with *Not*I and subcloned into the pBluescript KSII vector (Stratagene). The Genbank accession number for this sequence is AY055471.

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Abbreviations: COUP-TFI, chicken ovalbumin upstream promoter-transcription factor I; DBD, DNA-binding domain; EMSA, electromobility shift assay; DMEM, Dulbecco's modified Eagle's medium.

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Plasmids

COUP-TFI sequences used as probes for screening were obtained previously in our lab [20]. The sequences of the *COUP-TFI* promoter originally cloned into pBluescript, were subcloned into pGL3-basic (Promega) or a modified version of pGL3-basic that contains a consensus TATA box (pGL3-TATA). Vectors containing the coding sequences for ETV1, ERM and PEA3 were a generous gift from Y. de Launoit and L. Coutte (Institut Curie, Paris, France). Spi-1 was a gift from F. Moreau-Gachelin (Institut de Biologie, Lille, France). Ets-1 and Ets-2 were cloned by RT-PCR from rUGM cells.

Cell culture and transfection

HeLa cells were grown in 10% fetal bovine serum/Dulbecco's modified Eagle's medium (DMEM) (Gibco). The day before transfection, cells were passed onto six-well plates at approximately 5×10^5 cells per well. For transfection, both lipofectin (Gibco) and Fugene 6 (Boehringer) used at a 3 : 1 ratio to DNA gave similar results and both methods were used according to the manufacturer's instructions. Cells were collected 48 h later. Luciferase activity was measured in a luminometer (Monolight 2010, Analytical Luminescence Laboratory) according to the manufacturer's instructions. Protein content was measured using the Bradford reagent (Bio-Rad).

Electromobility shift assay

For EMSA (electromobility shift assay) studies, proteins were prepared (with or without [^{35}S]Met) from different DNA constructs using the TNT Coupled reticulocyte lysate system (Promega), according to the manufacturer's instructions. Probes for EMSA were end-labeled using α^{32}P-dCTP (ICN) and Sequenase enzyme (Amersham USB). EMSAs were carried out as follows: 2 µL of reticulocyte lysate were incubated for 10 min at room temperature with 1 µL of labeled probe (2–3 × 10^4 c.p.m.) and 10 pmol of dIdC in buffer H (20 mM Hepes, 1 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, 0.1% NP40, 1 mM spermidine, 5% glycerol). For competition experiments, 10- or 100-fold molar excess of unlabelled oligonucleotide were added to the incubation reaction. For supershift experiments, 125 ng of polyclonal anti-(Ets-1) Ig (Transduction Laboratories, San Diego, CA, USA) were added after the 10-min incubation and all tubes were then incubated on ice for an additional 15 min. Samples were subsequently loaded on a 5% acrylamide native gel and run at 30–50 mA. The gel was dried and exposed to Biomax film (Kodak).

The probe used (GTACCTCGAGCAGGAAGTTCGA) contained an Ets-1 consensus binding site (underlined).

Site-directed mutagenesis

To mutate specific base pairs within the *COUP-TFI* promoter, primers were designed within the sequence to be mutated. Each of these primers contained a restriction site for convenient subcloning. As three Ets binding sites (site A, at −490, site B, at −460 and site C at −420) were important, the GGAA sequence of each one was mutated to AGAA. PCR reactions were performed by standard methods using *Taq* polymerase (Promega). Deletions were carried out with mixed strategies using both PCR fragments with artificial restriction sites at the ends and DNA fragments obtained by restriction digestion. The oligos used for point mutations in the three ETS response elements are listed below (the ETS core binding sequences are in bold). A forward: CGGGTACCCTCCGTTTCCCACTTCTCG; A for Mut: CGGGTACCCTCCGTTTCTCACTTCTCG; B forward: CGGGGTACCTCCCTCTTCCCCGTCTTCTCGTTCGTTCG; B for Mut CGGGGTACCTCCCTCTTCTCCGTCTTCTCGTTCGTTCG; B reverse GAAGATCTCGAACGAACGAGAAGACGGGGAAGAGGGA; B rev Mut GAAGATCTCGAACGAACGAGAAGACGGAGAAGAGGGA; C rev GAAGATCTCAAGTCAGTCACAGGAAAAGAGC; C rev Mut GAAGATCTCAAGTCAGTCACAAGAAAAGAGC.

In situ hybridization

The 'B' domains of the ets-1 and ets-2 genes were used to prepare probes as described previously [21]. The N-terminus of ERM, ETV1 and PEA3 genes were used to prepare probes as described previously [22]. The full length cDNA of the *mCOUP-TFI* gene was used to prepare the COUP-TFI probe. The templates for probes were subcloned into pBluescript and RNA probes were prepared from linearized plasmid using T3 RNA polymerase (Promega) and 100 µCi [α-^{35}S]UTP (1000 Ci·mmol^{-1}, ICN). *In situ* hybridization was performed on 14.5-day-old mouse embryos as described previously [6,23].

RESULTS

Ets-1 activates the *mCOUP-TFI* promoter through a cluster of ETS response elements

To identify possible signals that activate the COUP-TFI gene transcription, we isolated approximately 6 kb of 5′ flanking sequences of the *COUP-TFI* gene (Fig. 1A). Reporter constructs containing different lengths of 5′ flanking sequences linked to a Luciferase gene were used on transient transfection studies. HeLa cells were chosen for these experiments because they express high levels of COUP-TFI [24]. The activity of the promoter was the same whether constructs containing 6, 4 or 0.73 kb of 5′ flanking sequences were used in the study. However, deletion of sequences between −734 and −387 resulted in a small but reproducible loss of promoter strength. Further deletion from −398 to −96, did not alter the activity. When the empty vector was studied, it had no significant activity (Fig. 1B). These results suggest that these two regions (−734 to −387 and −96 to +446 which includes promoter and 5′ UTR) are important for the *COUP-TFI* promoter activity (Fig. 1B). Within the distal region, a putative Ets-1 response element (site C, Fig. 2A) was identified by computer search for transcription factor binding sites [25]. Surrounding this element, we identified additional sequences that resembled ETS binding sites (Fig. 2). To assess whether Ets-1 regulates *COUP-TFI* promoter activity on this region, we cotransfected a reporter containing the −490/−259 fragment linked to a TATA-Luc reporter expression vector, in the presence or absence of an Ets-1 expression vector. Ets-1 was able to significantly

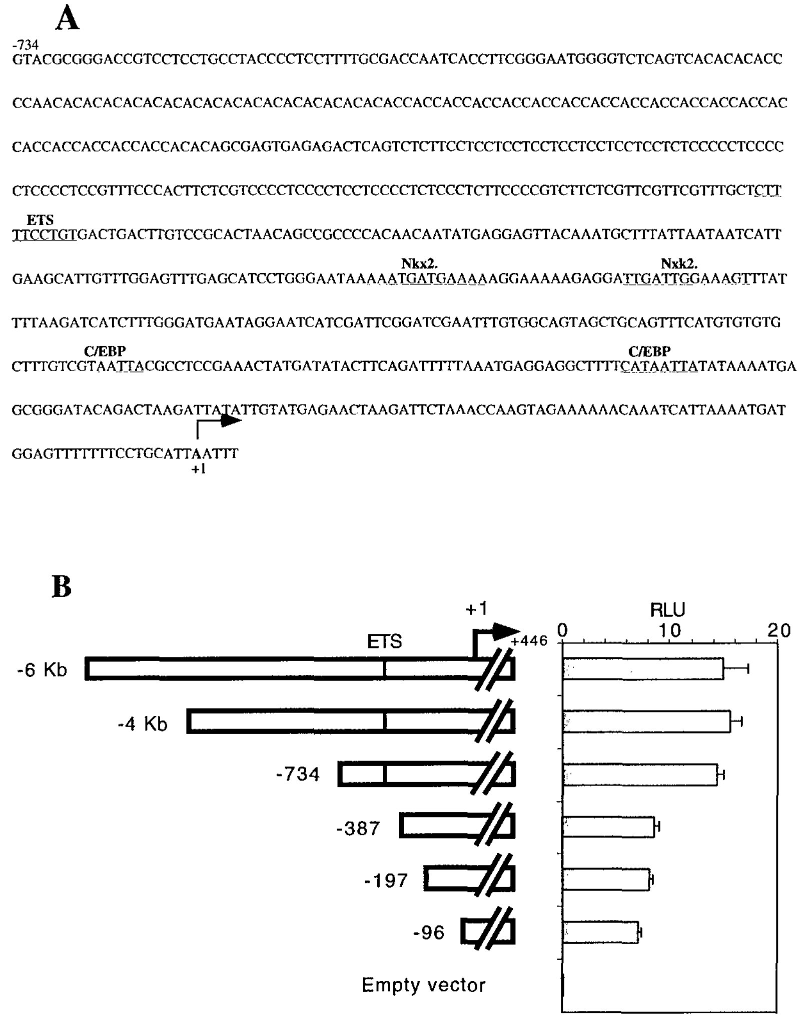

Fig. 1. General organization of the *mCOUP-TFI* promoter. (A) Sequence of the *mCOUP-TFI* promoter from −734 to +5. A putative Ets-1 binding site is underlined. Putative Nkx2. and c/EBP binding sites are shown underlined. The transcription initiation site is marked by an arrow. (B) Luciferase activity of 5′ deletions of the *mCOUP-TFI* promoter in transfected HeLa cells (0.25 μg DNA per well). A representative experiment performed in triplicate is shown. The putative Ets-1 binding site is marked as a lane.

activate the reporter gene containing this *COUP-TFI* promoter fragment. Then we subdivided that segment into two subfragments (−397/−259 and −490/−408), each carrying three putative binding sites. The subfragment −397/−259 is not responsive to Ets-1, while the −490/−408 subfragment is stimulated by Ets-1 to a similar extent as the −490/−259 fragment (Fig. 2A) in a dose-dependent manner (Fig. 2B). Interestingly, the best putative Ets-1 binding site sequence is located within this fragment.

The three putative Ets-1 response elements within −490 and −408 (ETS-RE) were then named A, B and C, in the order of 5′ to 3′. Point mutations (TTCC to TTCT) were introduced on each or combinations of the three putative sites. Mutations in one or two sites diminished Ets-1-dependent reporter activity (Fig. 2C), while mutations in all three sites abolished the response. These results indicate that these three Ets-1 response elements work in concert to achieve maximum Ets-1-dependent activation.

Ets-1 binds the ETS response elements in the *mCOUP-TFI* promoter

We examined whether Ets-1 was able to directly bind the response elements. On electromobility shift assays (EMSA) Ets-1 is able to bind to its DNA response element only if the auto-inhibitory domain is deleted [10]. Therefore, two truncations of the Ets-1 protein that lack part (Ets-1ΔCE, truncated from amino acids 280–331) or most (Ets-1ΔAE, truncated from amino acids 244–331) of the auto-inhibitory domain were made (Fig. 3A). These truncated proteins were shown to readily bind to ETS response elements [10]. To verify that our truncated proteins were active, we transfected HeLa cells with the −490/−408 luciferase reporter with increasing concentrations of Ets-1, Ets-1ΔCE or Ets-1ΔAE. These truncated forms of Ets-1 activate the *COUP-TFI* promoter (Fig. 3A). We next used *in vitro* translated proteins for DNA binding assays. The proteins were transcribed/translated using [^{35}S]Met and separated by PAGE. Figure 3B shows that Ets-1, Ets-1ΔCE and Ets-1ΔAE are all expressed to a similar level. On an EMSA using a consensus Ets-1 response element as a probe (Fig. 3C), no Ets-1 specific binding was observed when reticulocyte lysates were prepared with empty vector (lane 1) or with wild-type Ets-1 (lane 2). However, after addition of specific anti-(Ets-1) Ig to the wild-type Ets-1 lysate, a supershifted band was observed (lane 3). This was probably due to a stabilizing effect of the antibody on the Ets-1/DNA complex formation or to the possibility that the antibody may elicit a conformational change that

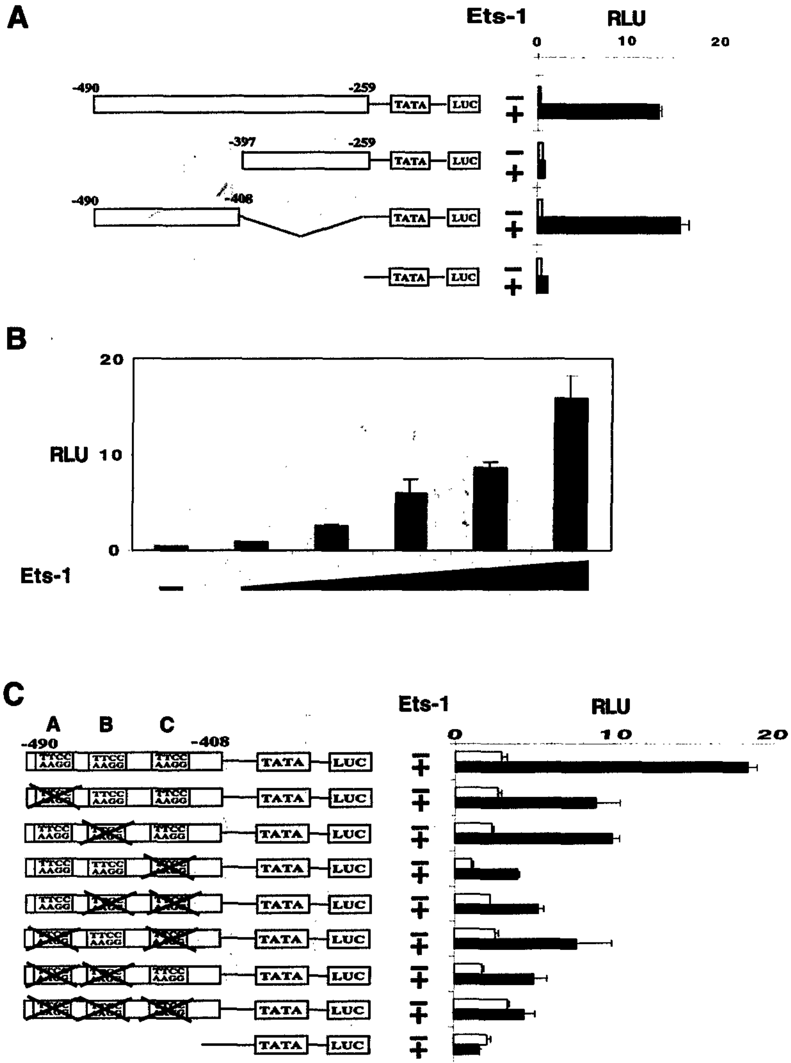

Fig. 2. Ets-1 induced the activity of the *COUP-TFI* promoter in HeLa cells. (A) Cells transfected with different portions of the promoter linked to a TATA box and a luciferase reporter gene (0.2 µg of DNA) with or without cotransfected Ets-1 expression vector (0.5 µg of DNA). (B) Dose–response of the Ets-1-dependent *COUP-TFI* promoter transactivation. The −490 to −408 reporter construct was cotransfected with increasing amounts of Ets-1 expression vector (0, 0.01, 0.04, 0.06, 0.1 and 0.2 µg). (C) Effect of single base pair mutations on the Ets-1 response of the −490/−408 fragment of the *COUP-TFI* promoter. The TTCC sequence was mutated to TTCT and the activation of these mutations was assessed by cotransfection with Ets-1 expression vector (0.2 µg of reporter, 0.5 µg of expression vector). Representative experiments performed in triplicate are shown.

at least partially relieves auto-inhibition. When lysate containing Ets-1ΔCE was added, a faint band corresponding to Ets-1ΔCE was detected (lane 4), and was completely supershifted by anti-(Ets-1) Ig (lane 5). When most of the auto-inhibitory domain was deleted (Ets-1ΔAE), a stronger band corresponding to Ets-1ΔAE was detected (lane 6), and it was completely supershifted by the antibodies (lane 7).

We next examined whether the ETS response elements in the *COUP-TFI* promoter were able to bind Ets-1 protein in a band-shift competition assay. The Ets-1 consensus binding site was used as a probe. Ets-1ΔAE was able to bind specifically (Fig. 3D, lane 3) and could be supershifted by an Ets-1 specific antibody (lane 2), but not by an unrelated antibody (lane 13). Increasing amounts (10 and 100-fold molar excess) of unlabeled site C oligos were able to compete for the binding of Ets-1ΔAE to the Ets-1 consensus binding site (lanes 4 and 5). In contrast, a mutation (TTCC to TACT) of site C was unable to do so (lane 6). Similar competition experiments were carried out with sites B (lanes 7, 8 and 9) and A (lanes 10, 11 and 12). Very weak competition could be detected with site A and B oligos, while oligos containing mutations of these sites did not compete at all (Fig. 3D). Taken together, these experiments suggest that Ets-1 is able to bind the *COUP-TFI* promoter preferentially at site C. This is not surprising because site C is the most closely related to the consensus Ets-1 binding site.

ETS factors colocalize with COUP-TFI on the developing mouse embryo

We performed *in situ* hybridization studies on mouse embryos with COUP-TFI and different ETS factors. Mouse embryos 14.5-days-old-were chosen for these experiments because the expression levels of COUP-TFI are high at this stage of development [1]. The expression patterns of COUP-TFI, Ets-1, Ets-2, ETV1 and PEA3 were studied (Table 1). There were several areas of coexpression of COUP-TFI and Ets-1: the mesenchyme of the bladder (Fig. 4A–D), the mesenchyme of the nasal septum (Fig. 4E–H), the cerebral cortex (Fig. 4I–L), the mesenchyme of vibrissae (Fig. 4M–P), spleen, and submandibular glands (Table 1). Ets-2 was found to colocalize with COUP-TFI on the mesenchyme of vibrissae (Figs 4M–N,Q–R) and submandibular glands (Table 1). PEA3 was found to colocalize with COUP-TFI in the cochlea, cerebral cortex and trigeminal ganglion (Table 1). ETV1 was found coexpressed with COUP-TFI on cells of the dorsal root ganglia and some

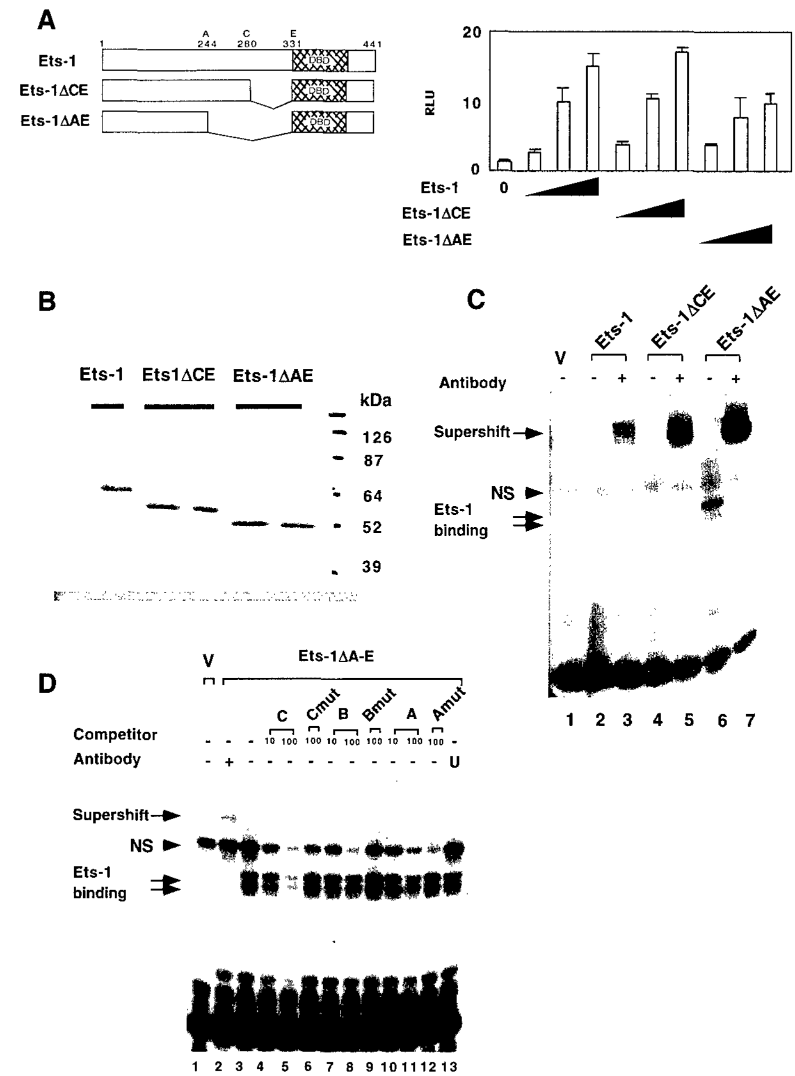

Fig. 3. Auto-inhibitory domain deleted Ets-1 is able to bind the *COUP-TFI* promoter. (A) Left panel, schematic view of the Ets-1 protein and the two deletions used in transfection and electromobility shift assay (EMSA) experiments. Right panel, HeLa cells were transfected with−490/−408 TATA reporter gene (0.2 µg) and increasing amounts of Ets-1, Ets-1ΔCE and Ets-1ΔAE (0.1, 0.2 and 0.3 µg). (B) Analysis of *in vitro* translated Ets-1, Ets-1ΔCE and Ets-1ΔAE constructs. *In vitro* translation was performed in the presence of ^{35}S-labeled methionine using a reticulocyte lysate system. The translated products were separated on a 10% SDS/PAGE and autoradiographed. For the deleted Ets-1, two vectors were used. (C) Electromobility shift assay (EMSA) of Ets-1, Ets-1ΔCE and Ets-1ΔAE on an Ets-1 consensus binding site. Lane 1 (V), vector control. Lanes 2 and 3, wild-type Ets-1 without or with anti-(Ets-1) Ig, respectively. Lanes 4 and 5, Ets-1ΔCE without and with antibodies, respectively. Lanes 6 and 7, Ets-1ΔA-E without and with antibodies. NS, nonspecific binding. (D) EMSA of Ets-1ΔAE on a consensus Ets-1 binding site and competition experiments (lane 1, vector control, lanes 2–13 contain Ets-1ΔAE). Lane 2, effect of anti-(Ets-1) Ig. For competition experiments, 10x and 100x molar excess (site C, lanes 4 and 5; site B, lanes 7 and 8; site A, lanes 10 and 11) or 100x molar excess (mutated site C, lane 6; mutated site B, lane 9; mutated site A, lane 12) of unlabeled oligonucleotide were used. Lane 13, addition of unrelated antibodies have no effect on Ets-1ΔAE binding.

Table 1. Expression of COUP-TFI, Ets-1, Ets-2, ETV1 and PEA3 mRNA in 14.5 day old mouse embryos.

Tissue	COUP-TFI	Ets-1	Ets-2	ETV1	PEA3
Cortex	+ + +	+	+/–	+	+
Cochlea	+ + +	+	+	+/–	+
Trigeminal ganglion	+	–	–	–	+
Dorsal root ganglia	+ +	+/–	–	+ + +	–
Mesenchima of mesonephric duct	+	+	–	–	–
Kidney	+ +	+	–	–	+
Stomach muscle	+ + +	+	+/–	+	–
Bladder	+ +	+	–	+	–
Spleen	+ +	+ +	+/–	–	–
Mesenchima surrounding genital tubercle	+ +	–	+	+	+
Mesenchima of the trachea	+	+	+/–	+/–	–
Spinal chord, mantle layer	+ +	–	–	–	+/–
Spinal chord, marginal layer	+	–	+	–	+/–
Submandibular gland	+ +	+	+	–	+
Mesenchima surrounding vibrissae	+ +	+	+	–	–

regions of the cerebral cortex (Table 1). In these tissues, ETS factors and COUP-TFI seemed to be localized in the same cell types. Figure 5 shows high magnification pictures of the signal for COUP-TFI and Ets-1 mRNAs in the nasal epithelium and COUP-TFI, Ets-1, and Ets-2 in the mesenchimal cells surrounding the vibrissae. These results indicate that COUP-TFI and ETS factors are colocalized in many regions of the developing mouse embryo.

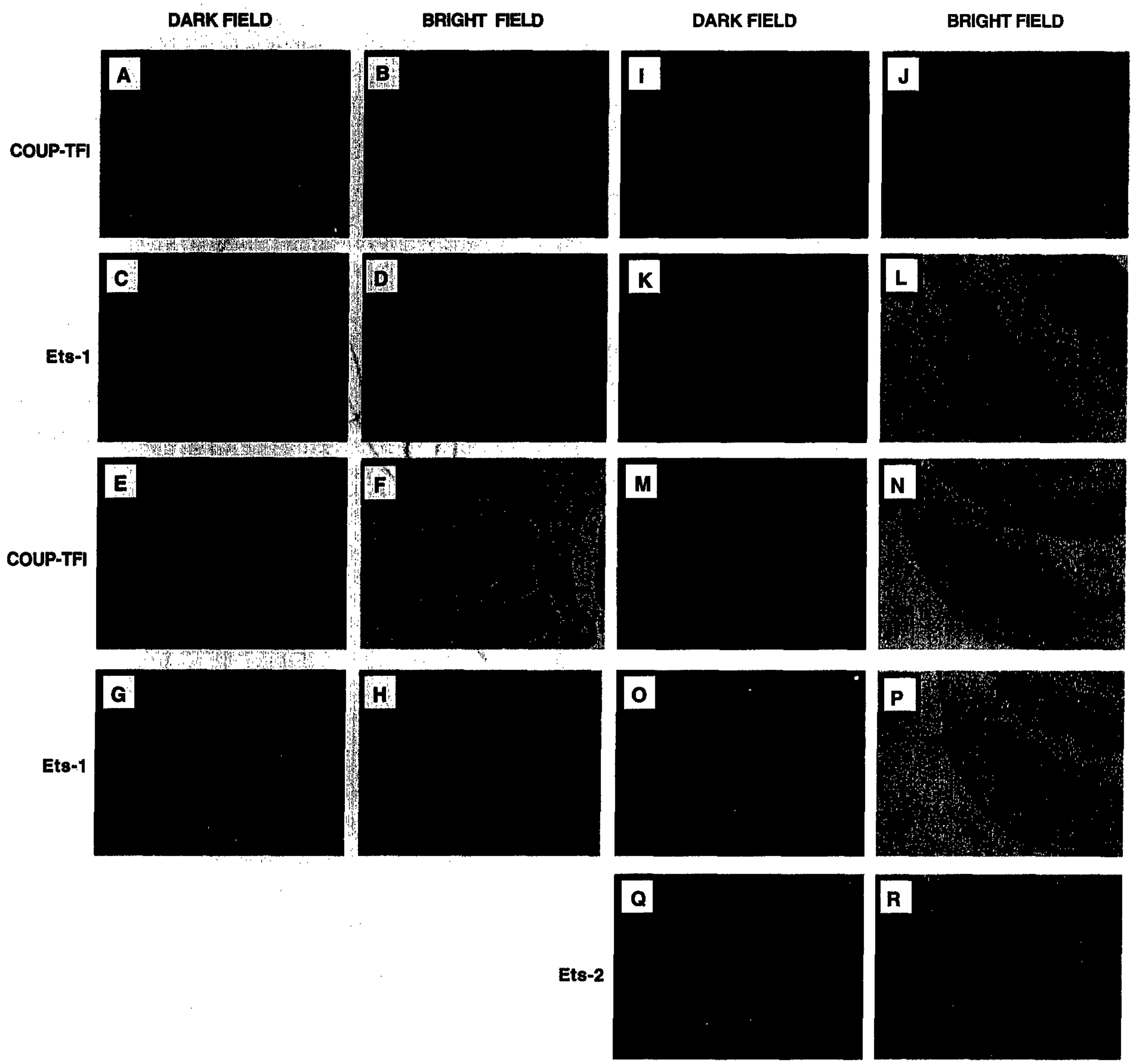

Fig. 4. Coexpression of *COUP-TFI* and *ETS* mRNAs. Embryos (14.5-day-old) were hybridized with probes for COUP-TFI (panels A, E, I and M), Ets-1 (panels C, G, K and O) and Ets-2 (panel Q). Hematoxilin counterstain is shown for each hybridization (panels B, D, F, H, J, L, N, P and R). (A–D), bladder; (E–H), nasal septum; (I–L), cerebral cortex; (M–R), vibrissae.

Other ETS factors are able to transactivate the *COUP-TFI* gene promoter

All ETS factors bind the same consensus core sequence GGAA/T, with surrounding bases conferring additional specificity [9]. Therefore, we cotransfected HeLa cells with the −490/−408 reporter gene and increasing amounts of expression vectors for ETS factors. Ets-2, Spi-1 and ETV1 were also able to activate the promoter (Fig. 6), consistent with the fact that the response elements for these proteins are very similar. Other ETS factors, namely ERM and PEA3, were also able to activate the *COUP-TFI* promoter but to a lesser extent (Fig. 6).

DISCUSSION

The last few years have seen a growing interest toward the ETS family and, as a result, the biological activities of some of these proteins have been studied. ETS factors are involved in processes such as development [26,27], tumor progression [8,28], specification of synaptic connectivity [29] and synapse-specific transcription [30]. Although there is a considerable body of research on ETS factors, only a few target genes have been identified.

In this paper, we have presented data indicating the coexpression of ETS proteins and COUP-TFI in the same tissues. Among these ETS factors, Ets-1, Ets-2, ETV1 and PEA3 are coexpressed with COUP-TFI in many different tissues of the developing mouse embryo suggesting that COUP-TFI may be a target gene of these factors. This would render a very complex pattern of activation of COUP-TFI as we showed that most ETS factors are able to activate the *COUP-TFI* promoter. Furthermore, the complexity of this system is also illustrated by the fact that ETS factors work together with accessory proteins [8]. Therefore, the final effect of a particular ETS factor on the promoter

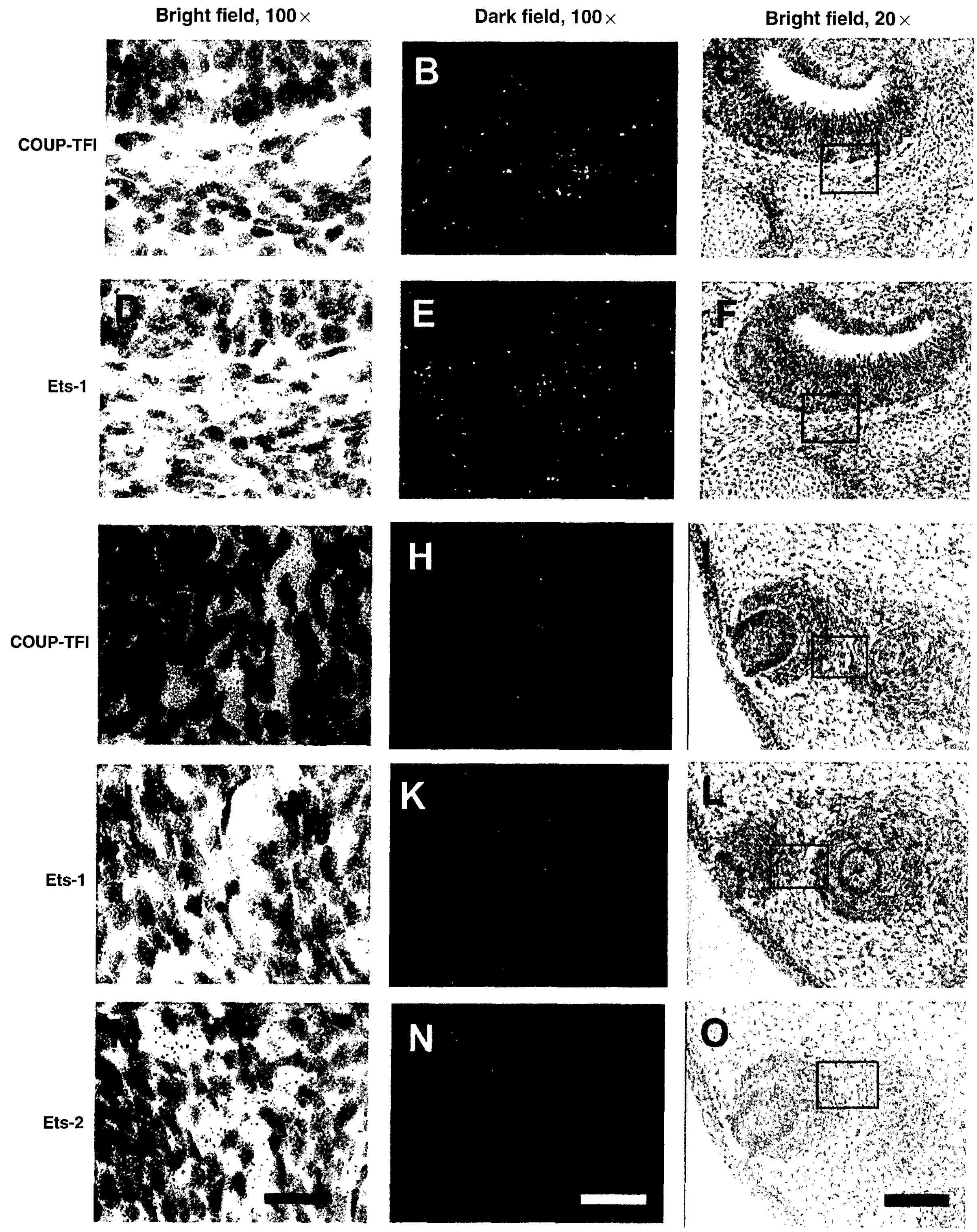

Fig. 5. Colocalization of COUP-TFI and ETS factors on e14.5 mouse embryos. Panels A–F, COUP-TFI and Ets-1 in nasal mesenchima. (A) COUP-TFI stained section, hematoxylin stained, at 100× magnification; (B) same region, seen on dark field; (C) 20× magnification of the same section. (D) Ets-1 stained section, hematoxylin stained, at 100× magnification; (E) same region, seen on dark field; (F) 20× magnification of the same section. Panels G–O, COUP-TFI, Ets-1, and Ets-2 in mesenchima surrounding the vibrissae. (G) COUP-TFI stained section, hematoxylin stained, at 100× magnification; (H) same region, seen on dark field; (I) 20× magnification of the same section; (J) Ets-1 stained section, hematoxylin stained, at 100× magnification; (K) same region, seen on dark field; (L) 20× magnification of the same section. (M) Ets-2 stained section, hematoxylin stained, at 100× magnification; (N) same region, seen on dark field; (O) 20× magnification of the same section. Black squares on panels C, F, I, L, and O denote the regions seen at 100×. Scale bars are 20 μm for 100× pictures and 200 μm for 20× pictures.

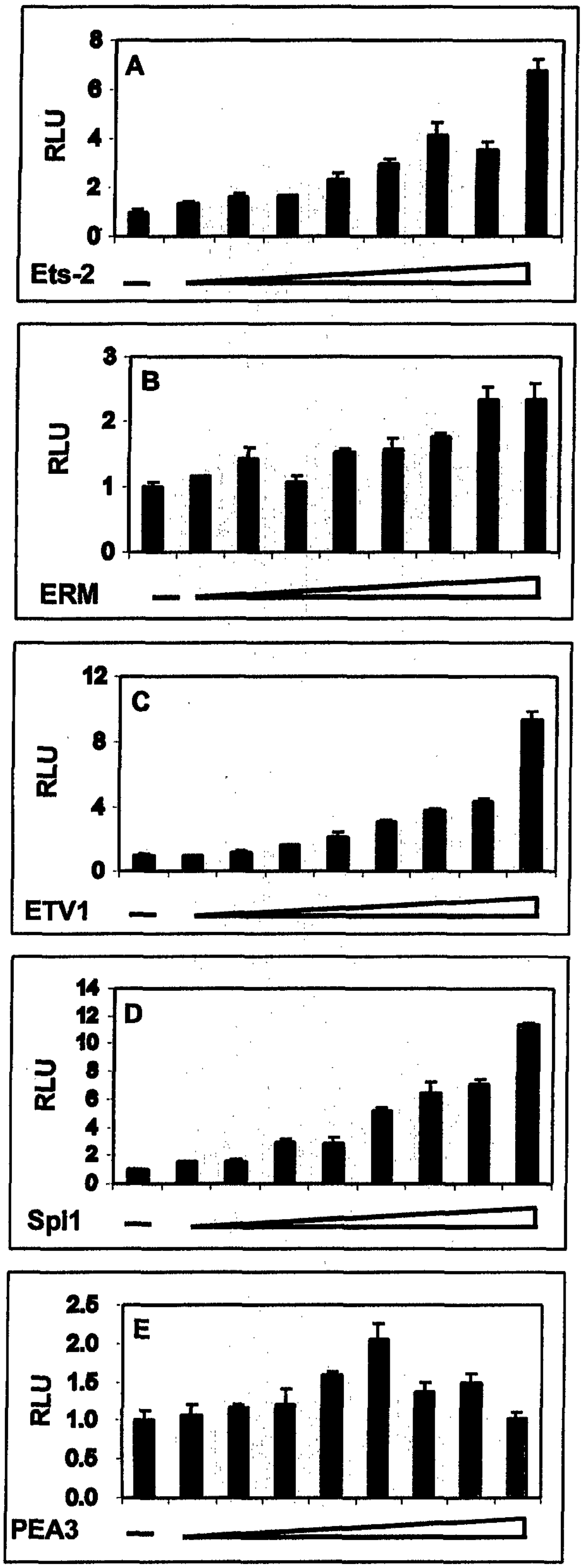

Fig. 6. Effect of other ETS family members on the *COUP-TFI* promoter. HeLa cells were transfected with the −490/−408 TATA reporter gene (0.2 μg) and increasing amounts (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8 and 1.0 μg) of ETS factors. (A) Effect of Ets-2. (B) Effect of ERM. (C) Effect of ETV1 (D) Effect of Spi-1 (E) Effect of PEA3. Representative experiments performed in triplicate are shown.

would also depend on the availability of these accessory factors.

When transfected in HeLa cells, the activity of the *COUP-TFI* promoter was about the same regardless of the size of the sequence used until the −387 construct was studied. The fragment between −734 and −387 seems to be responsible for half of the activity in these cells. We believe that this is the effect of endogenous Ets-1 or other ETS factors in HeLa cells. The activity drop is small probably because the reporter amount used in transfection experiments is in large excess and there might not be enough endogenous protein to reach full activation. In addition, we demonstrated that all three ETS sites must be occupied and this would be even more difficult when the ETS factors are present in a limiting amount. Finally, it is also possible that sequences closer to the initiation of transcription are responsible for a high basal activity.

In transfection experiments, all the ETS factors studied activated the *COUP-TFI* promoter, with Ets-1 showing the strongest effect. It is interesting to note that Ets-1 is also the factor that showed more regions of coexpression with COUP-TFI. Therefore, there may be a correlation between the level of coexpression and the extent of activation in transfected cells. The fact that all the ETS factors examined activated the *COUP-TFI* promoter is not really surprising. As stated earlier, all ETS factors recognize the same core motif. As the neighboring sequences also affect binding, the consensus binding site is not the same for all these proteins. Therefore, it is likely that the *COUP-TFI* promoter might have evolved to be more highly responsive to some members of the family, in this case Ets-1, as compared to others. The putative correlation between transactivation potential and mRNA coexpression supports this hypothesis. In addition, although we studied several ETS factors, there are many more, and it is possible that some other ETS factors also colocalize with COUP-TFI. Furthermore, the temporal expression of COUP-TFI and ETS factors change during development, which can alter the effect of ETS factors on COUP-TFI transcription [6,22].

In conclusion, we have identified the COUP-TFI transcription factor as a new putative target of ETS proteins. To answer whether Ets-1 or other ETS factors are true physiological regulators of COUP-TFI would requiere additional studies. This would be complicated because the ETS family has so many members, and we have demonstrated that different members are able to transactivate the *COUP-TFI* promoter. Therefore, the usual approach of studying the levels of COUP-TFI in ETS knock-out mice might not render the expected results, because compensation is very likely to occur.

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Advances in Brief

Loss of Heterozygosity Events Impeding Breast Cancer Metastasis Contain the *MTA1* Gene[1]

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Abstract

Breast cancer mortality is seldom attributable to the primary tumor, but rather to the presence of systemic (metastatic) disease. Axillary lymph node dissection can identify the presence of metastatic breast cancer cells and serves as a marker for systemic disease. Previous work in our laboratory determined that rates of loss of heterozygosity (LOH) of a 1.6-Mb region of chromosome 14q 31.2 is much higher in axillary lymph node-negative primary breast tumors than in axillary lymph node-positive primary breast tumors (P. O'Connell *et al.*, J. Natl. Cancer Inst., *91:* 1391–1397, 1999.). This unusual observation suggests that, whereas the LOH of this region promotes primary breast cancer formation, some gene(s) mapping to this 1.6-Mb region is rate-limiting for breast cancer metastasis. Thus, if primary breast cancers delete this region, their ability to metastasize decreases. To identify this gene(s), we have physically mapped this area of chromosome 14q, confirmed the position of two known genes and 13 other expressed sequence tags into this 1.6-Mb region. One of these, the metastasis-associated 1 (*MTA1*) gene, previously identified as a metastasis-promoting gene (Y. Toh *et al.*, J. Biol. Chem., *269:* 22958–22963, 1994.), mapped to the center of our 1.6-Mb target region. Thus, *MTA1* represents a strong candidate for this breast cancer metastasis-promoting gene.

Introduction

One of the strongest prognostic factors for cancer-free survival after treatment of the primary tumor is the presence or absence of local metastatic spread. For women with axillary lymph node-negative breast cancer, 90% survive more than 5 years after diagnosis. This is compared with a 70% 5-year survival for women with axillary lymph node-positive disease, and only a 20% 5-year survival for women with distant metastases (1). The development of the metastatic phenotype of a tumor cell involves a complicated series of events that include detachment of tumor cells from the primary tumor, invasion into and survival in the circulatory and lymphatic systems, extravasation, and induction of angiogenesis and growth at the metastatic site. The development of a genetic test that could predict the metastatic potential of a primary breast tumor would increase the effectiveness of breast cancer treatment.

Previous work in our laboratory involved LOH[3] analysis to compare DNA samples from paired normal and breast tumor tissues to examine whether specific genetic changes in primary breast cancer can serve as markers of metastatic potential (2). As expected, increasing rates of LOH were correlated with progressively higher stages of breast cancer (3, 4). Unlike all other 14 markers tested, LOH at marker D14S62 was much lower in metastases than in primary breast tumors. D14S62 LOH proved to be associated with node-negative primary cancers and thus with slower spread to distant sites. Higher resolution LOH studies narrowed this phenomenon to a 1.6-Mb region near marker D14S62 (2). Here we have assembled a physical map and identified a minimum tiling path of three YAC clones that span this region. One of the ESTs that mapped into this region in our study was *MTA1*, a gene previously shown to be highly expressed in both metastatic breast cancer cell lines and metastatic gastrointestinal carcinomas (5, 6).

Materials and Methods

YAC DNA Preparation and Mapping. CEPH YACs were selected for mapping of MTA1 by screening with D14S62 region markers or on the basis of available mapping information.[4] Total YAC DNA from each clone was purified as described previously (4). Each YAC clone was confirmed by PCR analysis using oligonucleotide primers for MTA1 and selected ESTs mapping into the region on the basis of the radiation hybrid mapping of chromosome 14 (Gene Map 1999 and the GDB). The primers used were a MTA1-expressed sequence tag (RH78599) designed by the Sanger Center and based on known human genomic chromosome 14 sequence.[5] The primer sequences were 5'GGTTCGGATTTGGCTTGTTA3', which is contained within a unique sequence of the MTA1 cDNA; and 5'CGTGGTTCTGGACAAGGG3', which is contained in the adjacent genomic sequence of MTA1. PCR was performed in a Gene Amp PCR system 9600 (Perkin-Elmer Corp., Norwalk, CT) using ~20 ng of YAC DNA in a volume of 50 μl in 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. A 20-μl volume of each product was electrophoresed in a 1% agarose gel, and PCR products were visualized by ethidium bromide staining.

BAC DNA Preparation and Mapping Analysis. BAC 76E12 was obtained from Research Genetics (Birmingham, AL). Total BAC DNA was purified according to the protocol supplied by the manufacturer. MTA1 was mapped to BAC 76E12 by PCR analysis using the same oligonucleotide primers for MTA1 as above. PCR was performed in a Gene Amp PCR system 9600 (Perkin-Elmer Corp.) using ~30 ng of BAC DNA in a volume of 50 μl in 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. A 10-μl volume of each product was electrophoresed in a 1% agarose gel, and PCR products were visualized by ethidium bromide staining.

Results

As part of our preliminary mapping studies of the region, we determined all of the ESTs that mapped into the target region according to the radiation hybrid-based NCBI Gene Map. A total of 12 ESTs, but no known genes, map to this 1.6-Mb metastasis-related region. However, we performed additional mapping analysis on the YACs used in the preliminary mapping analysis and confirmed two known genes and 13 ESTs that actually map to these YACs, rather to than their location indicated by the radiation hybrid-based NCBI Gene

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[3] The abbreviations used are: LOH, loss of heterozygosity; MTA1, metastasis-associated 1; EST, expressed sequence tags; NCBI, National Center for Biotechnology Information; GDB, Genome Database; YAC, yeast artificial chromosome; BAC, bacterial artificial chromosome.

[4] For example, Internet address: http://www-genome.wi.mit.edu; http://www.gdb.org.

[5] Internet address: http://www.sanger.ac.uk.

Map. Because of these findings, we included the region from marker D14S1066 to the telomere of chromosome 14 because of uncertainties inherent in radiation hybrid mapping to insure that we had complete coverage of the telomeric end of chromosome 14q. Approximately 392 ESTs map into this region, with 63 of these being previously identified genes. Table 1 summarizes the known genes in the region from D14S1066 to the telomere (including *MTA1*) considered to be possible metastasis candidate genes.

MTA1 had been mapped previously using radiation hybrids onto the NCBI Gene Map some distance away from our region of interest near the telomere of chromosome 14q. We had, however, discovered several ESTs thought to map elsewhere on chromosome 14 that were actually mapped to our target region. Because *MTA1* represented a promising candidate gene even though the gene map showed it to be outside our target region, we then tested PCR primers for the *MTA1* gene onto our physical map of the region detected by our LOH studies. We determined MTA1 mapped onto YACs 859d4 and 765h7 (Fig. 1). These mapping studies were subsequently confirmed when we mapped the gene onto BAC 76E12, which has a completed draft sequence that confirms its mapping onto YAC 859d4. The *MTA1* gene was therefore determined to map onto chromosome 14q in the vicinity of markers D14S62 and D14S51, or ~21 cM proximal to its previously reported location (see Fig. 1). Fig. 2, *A* and *B*, summarizes the gel-mapping data for MTA1. MTA1 was mapped onto the overlapping YAC clones 859d4 and 756h7. Fig. 2*B* indicates that MTA1 also maps to BAC 76E12, and its location to this sequenced BAC clone is confirmed by a BLAST search with MTA1 cDNA sequences.

Table 1 *Possible candidate genes in the 14q region of interest*

Nineteen known genes were determined to map into the area of interest on chromosome 14. Both the gene name and the GDB no. are given for identification.[a]

Gene name	GDB no.
CALM, calmodulin 1	9611304
PRSC1, protease, cysteine, 1	700617
CGHA, chromogranin A	119777
PI, protease inhibitor 1	120289
AACT, α-1-antichymotrypsin	118955
PCI, protein C inhibitor	134739
TCL1A, T-cell leukemia/lymphoma 1A	250785
CCNK, cyclin K	9957298
YY1, YY1 transcription factor	216988
CKB, creatine kinase, brain	120590
AKT1, V-akt murine thymoma viral oncogene	118989
EIF5, eukaryotic translation initiation factor 5	126411
IGHG3, immunoglobulin-γ 3	119339
MTA1, metastasis-associated 1	9955068
MARK3, MAP/microtubule affinity-regulating kinase	9315109
EMAPL, echinoderm microtubule-associated protein	6328385
KNS2, kinesin 2	304673
TRAF3, TNF receptor-associated factor 3	9836800
EEF10, eukaryotic translation elongation factor 10	216099

[a] More information on each gene can be found at http://www.gdb.org/.

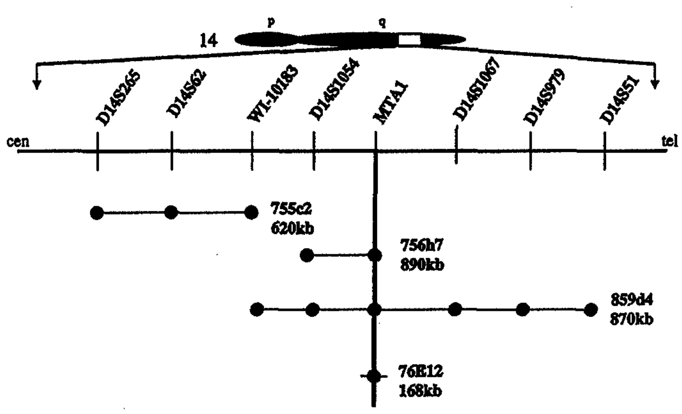

Fig. 1. YAC and BAC mapping of *MTA1*. A 1.6-Mb section of the region of interest was mapped, spanning from marker D14S265 to marker D14S51. The position of *MTA1* is shown relative to other markers on chromosome 14q. *MTA1* was determined to map onto both YAC 756h7 and YAC 859d4 and also to BAC 76E12.

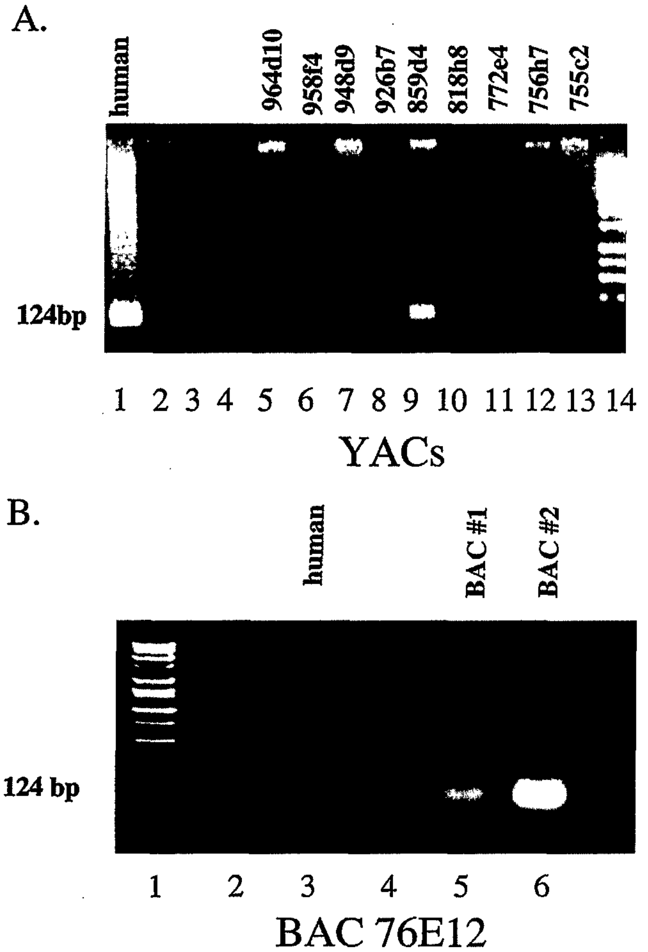

Fig. 2. Gel-mapping data on MTA1. *A*, PCR amplification of MTA1 primers on YAC clones spanning the metastasis gene target region (band shown in *Lanes 1, 9,* and *12*). Positive control was provided with 25 ng of human genomic DNA shown in *Lane 1*. *B*, PCR amplification of MTA1 primers on BAC clone 76E12. Two positives are shown in *Lanes 5* and *6* that represent two different preparations of the same BAC. Twenty-five ng of human genomic DNA was once again used as a positive control (*Lane 3*). Product size in both experiments was ~124 bp.

Discussion

MTA1 was previously identified as a metastasis-promoting gene overexpressed in both rat and human metastatic cell lines (5). The human *MTA1* gene was cloned and sequenced by Nawa *et al.* (7) in 2000. In 1994, the rat gene was cloned and sequenced by the same group (5). The expression of MTA1 in the human breast cancer cell line MDA-MB-231, a metastatic cell line, was determined to be approximately four times higher than its expression levels in the breast cancer cell line MDA-MB-468, which is nonmetastatic (5). The rat cell lines MTC.4, a benign line that remains phenotypically stable with prolonged passage, and the highly metastatic line MTLn3 were also tested for expression of *mta1* (the rat homologue). The expression level of *mta1* was found to be 4-fold higher in the MTLn3 line than in the MTC.4 line by Northern blotting (8). Different forms of cancer have also been shown to overexpress MTA1. Esophageal, colorectal, gastric, and pancreatic carcinomas have all been reported previously to express higher levels of MTA1 mRNA than paired normal tissues, and this overexpression correlated with the invasiveness or lymph node metastasis of each of the carcinomas (6, 9, 10).